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CONTRACT NO: DAMD17-90-C-0124

TITLE: AUTORADIOGRAPHIC DISTRIBUTION AND APPLIED PHARMACOLOGICAL CHARACTERISTICS OF DEXTROMETHORPHAN AND RELATED ANTITISSUE/ANTICONVULSANT DRUGS AND NOVEL ANALOGS

SUBTITLE: Receptor Binding of Dextromethorphan in Normal and Ischaemic Brain: A Comparison with Other Ligands

PRINCIPAL INVESTIGATOR: Professor Bowery
Kathryn Bevan

CONTRACTING ORGANIZATION: University of London
School of Pharmacy
Department of Pharmacology
29/39 Brunswick Square
London WCIN 1AX, England

REPORT DATE: November 1, 1991

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

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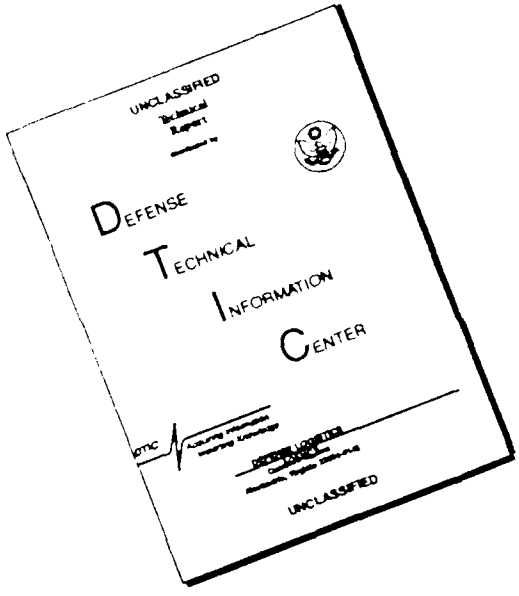

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1 November 1991	Annual Report (7/15/90 - 7/14/91)

4. TITLE AND SUBTITLE
Autoradiographic Distribution and Applied Pharmacological Characteristics of Dextromethorphan and Related Antitissue/Anticonvulsant Drugs and Novel Analogs

5. FUNDING NUMBERS
Contract No.
DAMD17-90-C-0124

6. AUTHOR(S)
Professor Bowery, Kathryn Bevan

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of London
School of Pharmacy, Department of Pharmacology
29/39 Brunswick Square
London WCIN 1AX, England

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Subtitle: Receptor Binding of Dextromethorphan in Normal and Ischaemic Brain: A Comparison with Other Ligands

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The objective of this project is to investigate the potential significance of the neuroprotective agent dextromethorphan, and its receptor, in normal and abnormal brain. Dextromethorphan is a non-opioid cough suppressant. It is as potent as codeine, but has the opposite steric configuration (i.e., it is dextrorotatory rather than levorotatory), and possesses none of the CNS pharmacology normally associated with opioids. The demonstration in 1981 of high affinity binding sites for dextromethorphan, which were distinct from any known neurotransmitter or neuromodulator sites, was followed by the discovery that dextromethorphan also possessed significant anticonvulsant and neuroprotective properties.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Drugs, RAI	
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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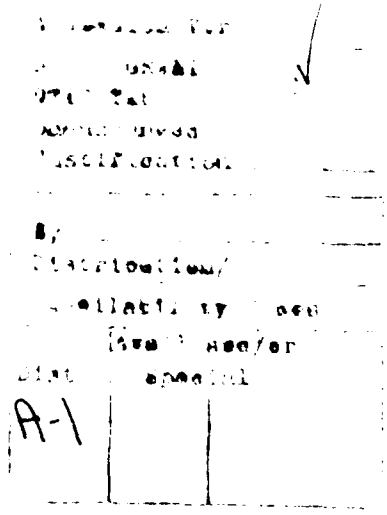


RECEPTOR BINDING OF DEXTROMETHORPHAN IN NORMAL AND ISCHAEMIC BRAIN: A COMPARISON WITH OTHER LIGANDS

The objective of this project is to investigate the potential significance of the neuroprotective agent dextromethorphan, and its receptor, in normal and abnormal brain. Dextromethorphan is a non-opioid cough suppressant. It is as potent as codeine, but has the opposite steric configuration (i.e. it is dextrorotatory rather than levorotatory), and possesses none of the CNS pharmacology normally associated with opioids. The demonstration in 1981 of high affinity binding sites for dextromethorphan, which were distinct from any known neurotransmitter or neuromodulator sites, was followed by the discovery that dextromethorphan also possessed significant anticonvulsant and neuroprotective properties.

The first phase of my project was to examine the characteristics of dextromethorphan's action at the receptor, using radioligand binding techniques. In general, these can be divided into two distinct but related methods. The first of these, autoradiography, uses brain sections, and allows the binding of the radioligand to be studied in situ. The advantage here is that the cell-cell interactions are still intact, and this should thus theoretically resemble the physiological state of the receptor. In addition, this technique allows the anatomical distribution of the receptor to be studied. However, there is one considerable disadvantage associated with autoradiography; the extremely slow throughput of material, and thus data, means that detailed quantitative information on the binding of several ligands is difficult to obtain. The other radioligand binding method, the now classical test-tube technique, allows data to be processed much more rapidly, and is therefore more suitable for studying more accurately the kinetics of binding together with receptor densities and affinities of ligands binding to multiple sites. However, this technique is also not without its disadvantages, and results can easily be misinterpreted. For example, preparation of the receptor material (i.e. homogenisation and sub-cellular fractionation) can expose binding sites not normally accessible to the radioligand or drug, and factors influencing binding (e.g. ions) may be removed during the preparation procedure. Overall, it was felt appropriate to make use of both techniques for this project; autoradiography for looking at the anatomical distribution of binding, and classical techniques for the quantitative assessment of receptor densities and understanding the pharmacology of the binding sites in terms of IC_{50}s or K_is.

During the first phase of my project I have attempted to reproduce and validate work previously carried out with dextromethorphan. So far, this has involved the preparation of receptor material (brain sections for autoradiography and membrane fractions for test-tube experiments); actual binding experiments using radiolabelled dextromethorphan and the appropriate receptor material, including preparation of various buffers and solutions, both according to established methods and also to optimise the various conditions used; and subsequently analysis and interpretation of data. Some of the preliminary results obtained are shown at the end of this report. Overall I feel I have mastered the various techniques and methodology involved, and am thus preparing to go on to the next phase of the project, which will be outlined below.

Dextromethorphan has been shown to be an effective neuroprotective agent, being active in both in vitro and in vivo models of ischaemia. It is widely accepted that the mechanisms of ischaemic neuronal damage involve glutamate receptors, of which the N-methyl-D-aspartate (NMDA) receptor is a sub-type. In addition to its own high affinity binding site, dextromethorphan also binds, albeit with lower affinity, to a site located on the ion channel associated with the NMDA receptor. This so-called phencyclidine (PCP) site is modulated by ligands which bind to the NMDA receptor. This includes NMDA itself, and also glycine and polyamines, which bind to sites distinct from the NMDA binding site and which are necessary for the channel to open. Antagonists at these binding sites have been shown to possess neuroprotective activity. Thus it would be of interest to compare the binding of dextromethorphan (and a series of structurally related analogues) to the dextromethophan high affinity binding site, as measured using [3H]dextromethorphan, and also to both the PCP and glycine sites, using [3H]MK-801 and [3H]glycine respectively. To this end I have developed radioligand binding assays for both glycine and MK-801 in the same manner as for dextromethorphan.

So far the mechanism of the neuroprotective action of dextromethorphan has not been established. It may be via its own high affinity site, or it may be via its action at the PCP site (either directly, or via metabolism to dextrorphan, which has a higher affinity for the PCP site than dextromethorphan). Thus it is proposed to examine the binding of dextromethorphan not only to normal brain, but also to brains which have been subjected to an ischaemic insult. This will be done using the "middle cerebral artery occlusion" model in the rat, which has been shown to cause a reproducible focal cortical infarct, and which is widely used to study the neuroprotective activity of drugs. The binding of dextromethorphan will be compared with that of other ligands associated with neurotoxicity and neuroprotection. These will include glycine and MK-801, and also the opioid antagonist naloxone, as dextromethorphan has at least one pharmacological effect in common with certain opioids in its antitussive action and naloxone has been shown to possess neuroprotective properties. Binding will be studied both in normal brain, and in ischaemic brain 72 hours post-occlusion, after which time infarct formation has been shown to be near maximal. If differences are apparent, a time course from 0-72 hours looking at binding versus infarct formation will be examined in more detail. As the middle cerebral artery occlusion model is a complicated one, agreement has been reached with the Stroke Project at Pfizer Central Research, for the appropriate training in this technique.

To follow on from this work, it is proposed to attempt to correlate neuroprotective activity with levels of these agents achieved in the brain after in vivo administration. Generally, if a compound binds to more than one site with different affinity, it is assumed that the high affinity site is more relevant. However, after in vivo administration, brain levels may be achieved such that the relevant action of the agent to elicit any given effect is at lower affinity sites. This may especially be the case with dextromethorphan. Thus brain levels of the above agents after neuroprotective doses will be determined subsequent to administration of radiolabelled tracer in vivo, followed by purification of the radiolabel using TLC in vitro.

Results

Figure 1 shows a typical displacement curve for [3H]DM in guinea pig brain sections (10µM). Using 20nM [3H]DM, incubating for 20 minutes at 23°C, and washing 3x5min in ice cold Tris-HCl containing 0.01% Triton X-100 and 100mM choline chloride, the IC_{50} for dextromethorphan was found to be 380nM. The presence of more than one binding site is indicated by the low slope observed with this curve.

Figure 2 demonstrates that the binding of [3H]DM to guinea pig brain crude homogenate is proportional to the amount of protein present. Specific binding is defined as total minus non-specific (in the presence of 100µM DM) and accounts for 46-67% of total binding, which is expressed here in dpm.

Figures 3 and 4 show the binding of [3H]DM in sub-cellular fractions of guinea pig brain. Binding of DM to crude (unpurified) homogenates is generally found to be of low affinity (~10µM), reflecting the greater capacity of the low affinity site for dextromethorphan. The fractions were prepared according to the method of De Robertis et al (1962). The binding assay was performed by incubating 50µl of [3H]DM (4nM) with 400µl of protein and 50µl of either buffer (Tris-HCl, pH 7.4 @ 23°C) or increasing concentrations of cold DM (1nM-100µM). Non-specific binding was defined with 1mM DM. Highest affinity binding was found in the microsomal fraction, with lower affinity binding in synaptosomal and mitochondrial fractions. However, binding in all of these fractions appears to be biphasic, indicating more than one site is present, although this could be due to incomplete purification of each fraction during the centrifugation procedure.

Other experiments were performed to optimise the conditions used in the [3H]DM binding assay (data not shown). Association experiments showed that equilibrium was reached within 1 minute at 23°C, and remained stable for at least 30 minutes. Non-specific binding of [3H]DM to the glass-fibre filters proved to be a problem. Presoaking the filters in Tris-HCl containing either Triton X-100/choline chloride or PEI did not improve the ratio of specific to non-specific binding, or using GF/C or GF/F filters instead of GF/B, or including 1mM DM in the presoak. These results are in contrast to those reported (claiming that addition of choline chloride/Triton X-100 to the filter presoak and wash buffers reduces binding of [3H]DM to the filters); the reason for this is not known.

In addition to the dextromethorphan binding assay, assays for glycine and MK-801 have also been developed. Both use an extensively washed rat crude cortical synaptosomal (buffy coat) preparation. For the glycine assay, 50µl of [3H]glycine (20nM) was incubated with 400µl of protein and 50µl of either buffer (50mM Tris-citrate, pH 7.7 @ 4°C) or increasing concentrations of cold glycine (1nM-100µM), for 20 minutes at 4°C. Non-specific binding was defined with 0.1mM glycine. The reaction was terminated by dilution with 3ml ice-cold Tris-citrate buffer followed by rapid filtration through GF/C filters presoaked ~2hr in Tris-citrate buffer containing 0.1% PEI/1mM glycine to reduce non-specific binding to the filters. All of the preceding steps were performed in the cold room, and the whole separation procedure was carried out as rapidly as possible (<10sec) to minimise dissociation of the ligand from its receptor. For the MK-801 assay, Tris-HCl (pH 7.4 @ 23°C) was the buffer used, 1nM [3H]MK-801 was incubated with the membranes for 1 hour at 23°C. 10µM MK-801 was used to define non-specific binding, and filtration was through GF/B filters

presoaked in 5mM Tris-HCl. The effects of added glutamate at 10μM were also studied. Glutamate increased the specific binding of [^{3}H]MK-801 by nearly 60% (from 5863 to 9178 dpm; no effect on non-specific binding), while not significantly affecting glycine binding.

Experiments are also in progress to examine the effects of the carbetapentane and dextromethorphan analogues, obtained from Dr Newman at NIDA-ARC, in these assay models.

As soon as the final data from these analyses have been made the results will be conveyed to Dr Tortella and Dr Newman.

Fig. 1

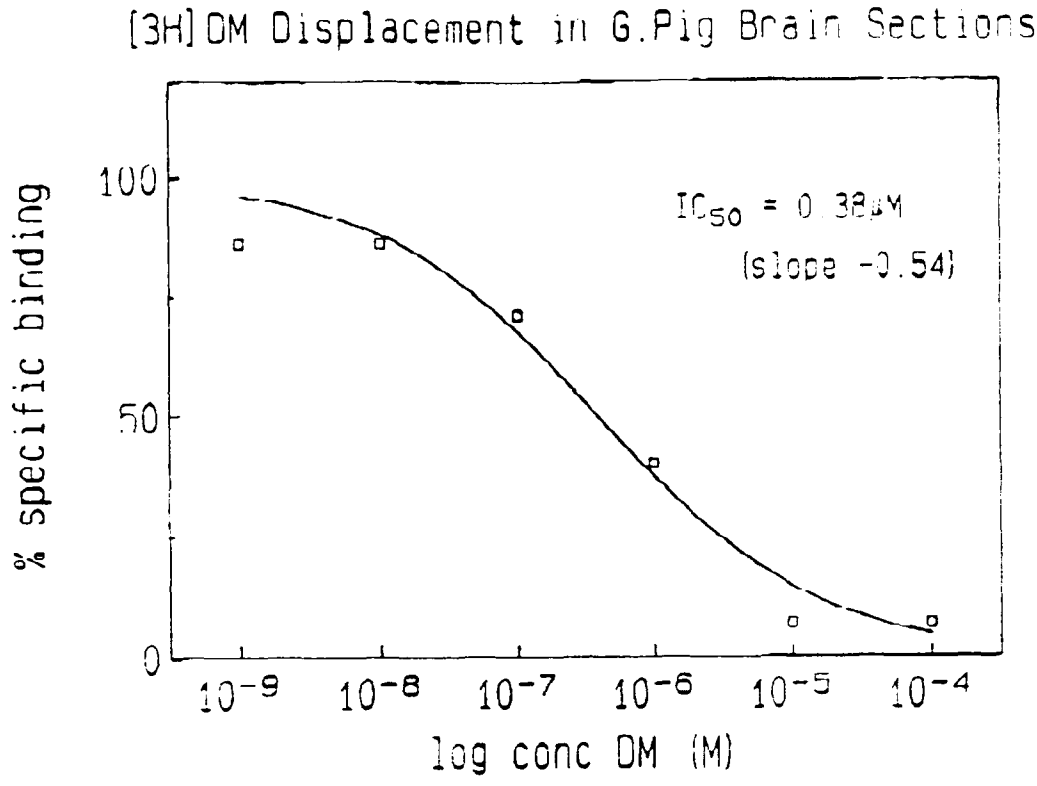

[3H] DM Displacement in G.Pig Brain Sections
% specific binding
100
50
0
IC_{50} = 0.38μM
(slope -0.54)
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
log conc DM (M)

Fig. 2

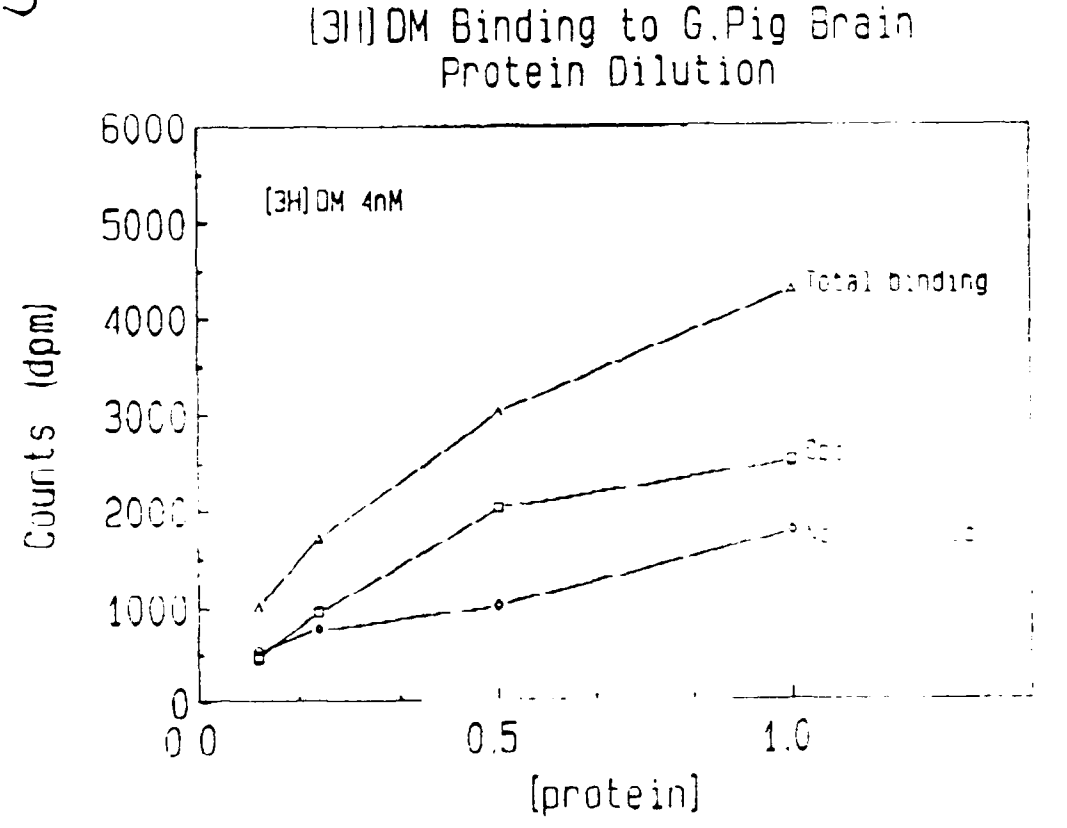

[3H] DM Binding to G.Pig Brain
Protein Dilution
[3H] DM 4nM
Counts (dpm)
6000
5000
4000
3000
2000
1000
0
Total binding
0
0.5
1.0
[protein]

Fig 3

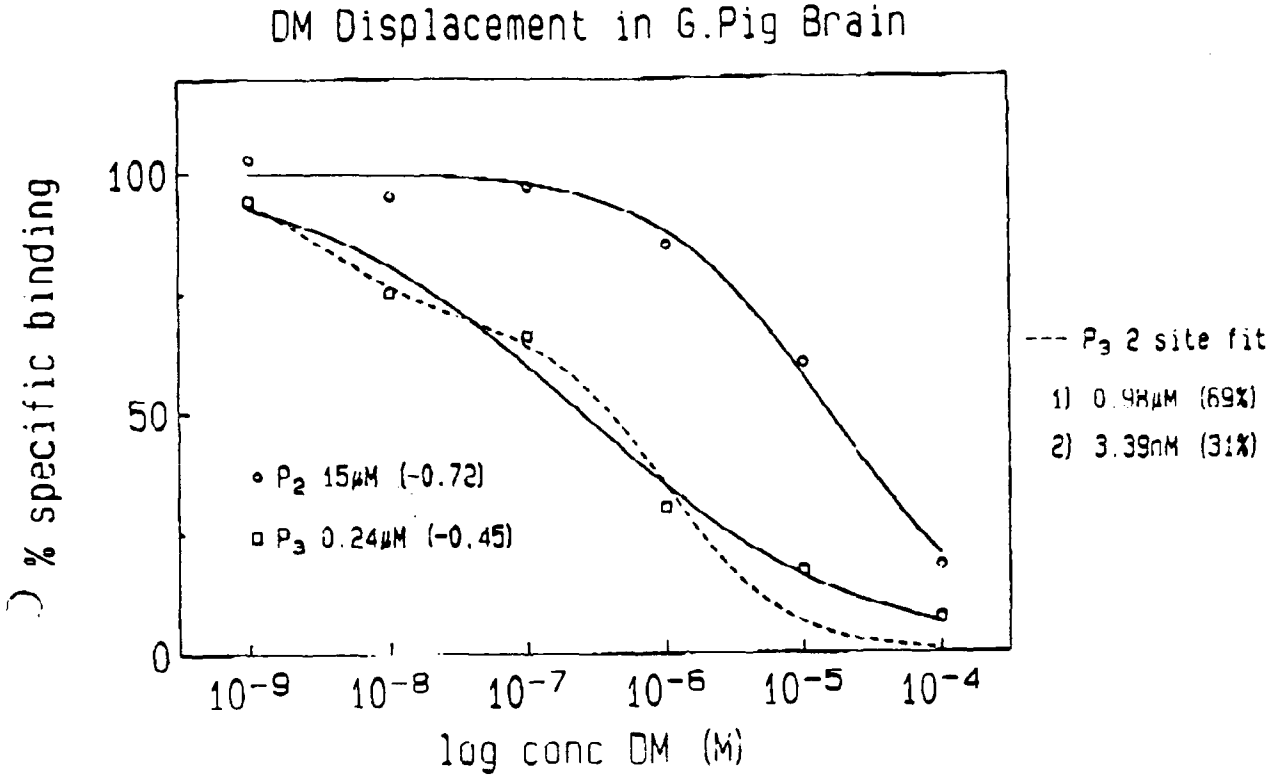

DM Displacement in G.Pig Brain
% specific binding
100
50
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
log conc DM (M)
P_2 15μM (-0.72)
P_3 0.24μM (-0.45)
--- P_3 2 site fit
1) 0.98μM (69%)
2) 3.39nM (31%)

Fig 4.

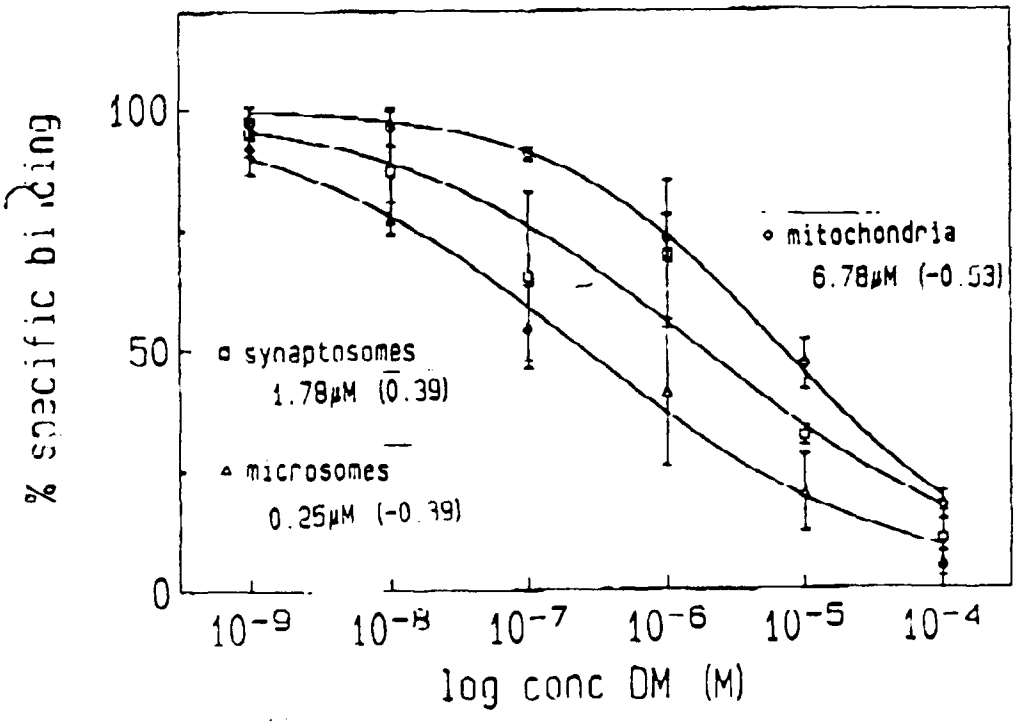
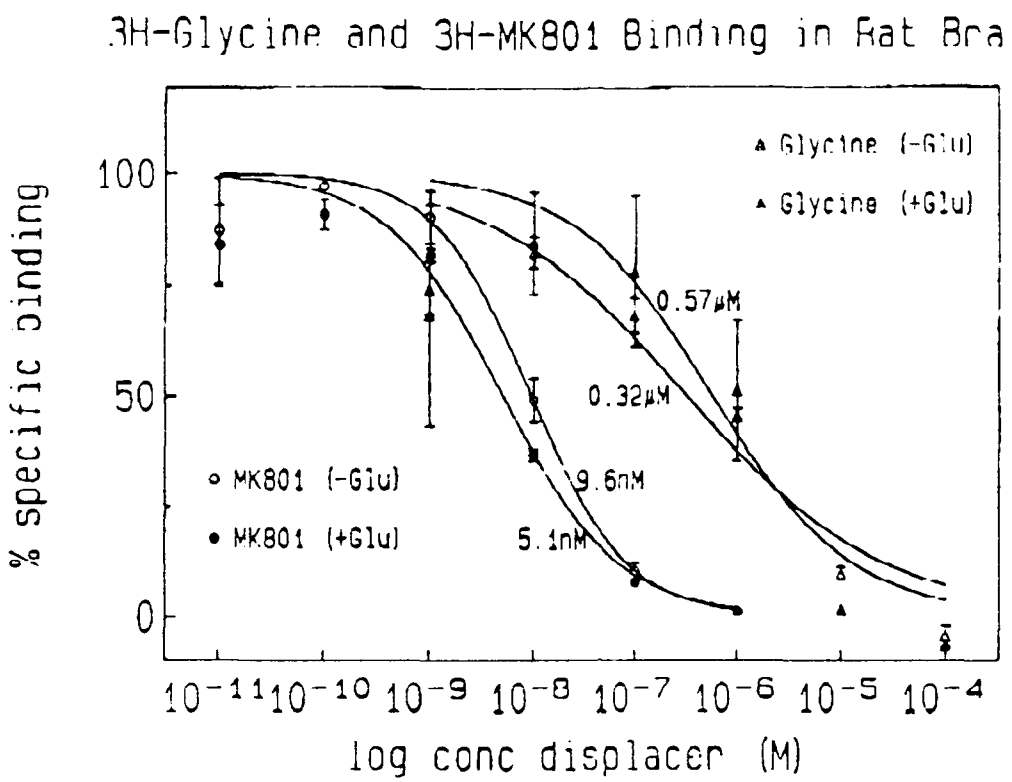

% specific binding
100
50
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
log conc DM (M)
mitochondria
6.78μM (-0.53)
synaptosomes
1.78μM (0.39)
microsomes
0.25μM (-0.39)

Fig 5.


3H-Glycine and 3H-MK801 Binding in Rat Brain
% specific binding
100
50
0
▲ Glycine (-Glu)
▲ Glycine (+Glu)
0.57μM
0.32μM
9.6nM
5.1nM
○ MK801 (-Glu)
● MK801 (+Glu)
10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4
log conc displacer (M)